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PRINCIPAL INVESTIGATOR: Gang Yao

CONTRACTING ORGANIZATION: University of Missouri
Columbia, MO 65211

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Gang Yao

E-Mail: yaog@missouri.edu

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14. ABSTRACT

Pupillary light reflex (PLR) refers to the involuntary response whereby the pupil size changes in response to a short flash light. In this project, we will evaluate the atypical dynamic pupillary light reflex (PLR) observed in children autism. In addition, we will develop an integrated PLR-
fMRI protocol for imaging PLR associated brain activities. At the end of the 2nd project year, we have tested pupillary light reflex (PLR) and heart rate variability (HRV) in 304 participants including 152 children with ASD (the "ASD" group), 116 children of typical development (the "TD" group), and 36 children with other development disorders (the "NDD" group). The results showed that the ASD group had significantly longer PLR latency, reduced relative constriction amplitude, and lesser constriction/ redilation time than those of the TD group. Similar atypical PLR parameters were observed in the NDD group. A significant age effect on PLR latency was observed in children younger than 9 years in the TD group, but not in the ASD and NDD groups. Atypical HRV parameters were observed in the ASD and NDD groups. A significant negative correlation existed between the PLR constriction amplitude and average heart rate in children with an ASD, but not in children with typical development. We also developed an integrated fMRI/PLR protocol and have obtained fMRI data for 25 adolescents with ASD and 25 typically developing adolescents without ASD.

15. SUBJECT TERMS

None provided.

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Table of Contents

INTRODUCTION

In this project, we propose to further evaluate the atypical dynamic pupillary light reflex (PLR) observed in children autism. PLR refers to the involuntary response whereby the pupil size changes in response to a short flash light. There are two specific tasks in this 3-year project. In Task #1, we propose to test 200 human subjects including 100 children with autism ("ASD" group), 65 typically developing children ("TC" group), 35 children with early brain dysfunction unrelated to autism ("MR" group). In addition to the PLR test, each participant will be assigned a score as the likelihood to have ANS dysfunctions based on an extensive medical evaluation. The heart rate variability (HRV) data will also be obtained as a reference. In Task#2, we will develop a new integrated PLR/fMRI test and study PLR correlated fMRI in a total of 50 human subjects (25 children with autism and 25 typically developing children). We will test the hypothesis that the observed atypical PLR latency in individuals with autism is associated with abnormal cerebellum functions.

BODY:

During the preceding 24 months, we have tested pupillary light reflex (PLR) and heart rate variability (HRV) in 304 participants including 152 children with autism, 116 children of typical development, and 36 children with other development disorders. As described below, the current results confirmed atypical PLR and revealed significant different HRV in children with autism. We have also obtained fMRI data for 25 adolescents with ASD and 25 typically developing adolescents without ASD.

Description of progress in Task #1:

Protocol

The protocol has been described in details elsewhere (Daluwatte et al., 2012). We measured the pupillary light reflex (PLR) under both dark-adapted and light-adapted conditions in each participant. The recorded pupil images were automatically processed to extract the pupillogram (change in pupil size with time shown in Fig.1). The following PLR parameters were measured: the initial pupil diameter D_0, the maximal constriction diameter D_m, the PLR latency t_L (interval between stimulus onset and beginning of constriction), the constriction time t_C (interval between constriction onset and the maximal constriction), and the recovery time t_R (interval between the maximal constriction and the recovery to half of the maximal constriction). The relative constriction (in percentage) was calculated as $(D_0^2-D_m^2)/D_0^2$.

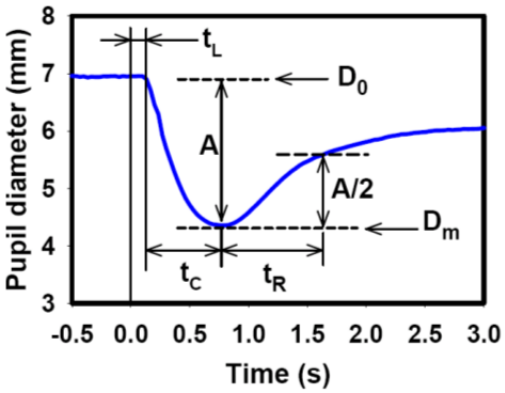

Fig.1. An illustration of the pupillogram and extracted PLR parameters. Please see text for explanations of each parameter.

We also monitored the participant's heart rate during the entire test.A remote heart rate monitoring device (Polar RS800CXTM, Polar Electro Oy, Finland) was chosen to record QRS interval in real time. A chest strap with sensor and wireless transmitter attached is wrapped around the participant's chest. The heart beat QRS signals transmitted from the chest strap is received and recorded by a watch-like device. The HRV dataset includes five standard HRV

measures: standard deviation of normal-to-normal RR intervals (SDNN), root mean square successive differences (RMSSD), low-frequency component LF (0.04- 0.15Hz), high frequency component HF (0.15-0.4Hz), and the ratio of LF/HF. The values are reported during five different time segments in the PLR test: before PLR test, during light-adapted PLR test, during dark-adaptation, during dark-adapted PLR test, and after all PLR test.

In addition to PLR and HRV, we collected a comprehensive questionnaire on each participant's medical history including autonomic nervous system (ANS) functions, fever history, sleep disorders, sensory profile and medication.

Participants

A total of 152 children with an ASD participated in this study (referred to as the "ASD" group). The ages ranged from 5 to 19 years with an average age of 10.7 ± 3.4 years; the group consisted of 135 boys (10.9 ± 3.5 years) and 17 girls (9.8 ± 2.6 years). Of the 152 participants, 145 were patients receiving clinical services at the University of Missouri Thompson Center for Autism and Neurodevelopmental Disorders, an interdisciplinary academic medical center specializing in diagnosis and treatment of ASD. The remaining 7 children were diagnosed using a variety of measures, which were reviewed by the authors to confirm the ASD diagnosis.

Among the 152 children with ASD, 86 were diagnosed with classic autism, 32 with Asperger's Syndrome, and 34 with pervasive developmental disorder-not otherwise specified (PDD-NOS). Seventy children in the ASD group had taken one or more medications (includes stimulants, atypical antipsychotics, serotonin reuptake inhibitors, antihistamines, antiepileptics etc.) within 48 hours before the PLR test (referred to as the "w/med" group). The remaining children had not taken medication (referred to as the "w/o med" group).

A sample of 116 typically developing healthy children between 6 and 17 years of age without known visual, neurological, or cardiovascular problems comprised a typically developing comparison group (referred to as the "TD" group). Nine children who had a sibling with ASD were excluded from the data analysis. Thus 107 children (mean age = 10.9 ± 2.9 years) were included in the TD group, which consisted of 79 boys (mean age = 11.1 ± 3.1 years) and 28 girls (mean age = 10.6 ± 2.4 years). All participants in the TD group scored below the clinical cutoff (<15) on the Social Communication Questionnaire Lifetime (Eaves et al. 2006) (mean score = 2.3± 2.8). None of the TD participants had taken medications within 48 hours before the PLR test.

A sample of 36 children ranging in age from 5 to 17 years of age (mean age = 9.9 ± 3.0 years) with intellectual disabilities due to other neurodevelopmental disorders (NDDs) also participated in this study. This group, referred to as the "NDD" group, included 27 boys (mean age = 10.0 ± 3.1 years) and 9 girls (mean age = 9.7 ± 2.6 years). This group included Down syndrome (7), Fragile X syndrome (5), Neurofibromatosis Type One (1), Prader-Willi syndrome (1), and the remainder with idiopathic intellectual impairment. Nineteen children in the NDD group were on medications similar to those described above for the ASD group.

Intelligence quotient (IQ) scores were available for all participants with the exception of 16 children in the ASD group. The vast majority of IQ scores were derived from the Ravens Progressive Matrices (RPM) (Raven 1996) (n = 95 ASD, 107 TD, and 36 NDD). The remainder were derived from the Wechsler Abbreviated Test of Intelligence (n = 12 ASD), Differential Abilities Scale – 2nd Edition (n = 15 ASD), Leiter International Performance Scale – Revised (n = 9

ASD) and Stanford-Binet Intelligence Scales – Fifth Edition (n = 5 ASD). For purposes of later analysis of the relationship between overall intellectual ability and PLR parameters, participants were categorized into either the "Low IQ" group or the "High IQ" group. An IQ equivalent of 80 or higher (9.1 percentile) was used to designate a child with normal-to-above normal intelligence (Wechsler 1991). Thus, the 9.1 percentile was used for those who were assessed with the RPM, and a threshold score of 80 was used for children who had been assessed by other IQ tests. Distributions of the IQ subgroups and medication status of participants are shown in Table 1.

Table 1. Distribution of IQ and medication use in TD, ASD and NDD groups.

	Group	IQ	w/o med	w/med
TD		High-IQ	**105**	**0**
		Low-IQ	**2**	**0**
ASD		High-IQ	**52**	**36**
		Low-IQ	**25**	**23**
ASD diagnosis	Asperger	High-IQ	12	12
		Low-IQ	2	1
	Autism	High-IQ	25	15
		Low-IQ	21	16
	PDD-NOS	High-IQ	15	9
		Low-IQ	2	6
NDD		High-IQ	**10**	**9**
		Low-IQ	**7**	**10**

High-IQ: IQ score of 80 or higher (at or above 9.1th percentile)
Low-IQ: IQ score lower than 80 (below 9.1th percentile)

We were not able to acquire HRV in 9 children in the ASD group, 1 in the TD group, and 1 in the NDD group because the participants declined to wear the heart rate sensor. A malfunction of the heart rate sensor resulted in missing HRV data in 2 other children in the ASD group. In addition, PLR images of 2 children in the ASD group, 1 child in the TD group, and 3 children in the NDD group could not be processed because of excessive eye movement or closure during the test.

Data analysis

The Kolmogorov-Smirnov test was used to verify normal distributions of all measured PLR and HRV parameters. For each PLR and HRV parameter, the Analysis of Covariance (ANCOVA) using the PROC MIXED procedure in SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA) was applied to examine the effects of group (TD, ASD, and NDD), age, and test conditions (stimulus intensity/test phase and time of day of the test). Follow up analysis of variance (ANOVA), multivariate analysis of variance (MANOVA), and t-tests with Bonferroni correction were used appropriately to confirm effects revealed by the ANCOVA model. ANOVA model was applied to study the effects of IQ (High IQ and Low IQ) and medication ("w/o med" and "w/med") in the ASD and NDD groups, and the effect of ASD diagnosis (classic autism, Asperger's, and PDD-NOS) in the ASD group. The method reported by Steyn and Ellis (2009) was applied to evaluate effect size ($\hat{\eta}^2_{\Lambda,r=1}$) for group differences using MANOVA. An $\hat{\eta}^2_{\Lambda,r=1}$ value of 0.02, 0.13 and 0.26 was considered as a small, medium and large effect, respectively (Steyn and Ellis, 2009). Pearson product moment correlation was applied to study correlation between PLR parameters and

HRV parameters. A p value <0.05 was considered significant.

Results

The ASD and NDD groups had a significantly longer latency ($F_{4,229}$ = 23.24, p < 0.0001, $\hat{\eta}^2_{\Lambda,r}$ = 0.28 for ASD and $F_{4,130}$ = 21.69, p < 0.0001, $\hat{\eta}^2_{\Lambda,r}$ = 0.38 for NDD) and lesser relative constriction amplitude ($F_{4,231}$ = 4.47, p = 0.002, $\hat{\eta}^2_{\Lambda,r}$ = 0.06 for ASD and $F_{4,130}$ = 3.74, p = 0.007, $\hat{\eta}^2_{\Lambda,r}$ = 0.08 for NDD) than those of the TD group for all testing conditions. The ASD group also had a shorter constriction time (MANOVA $F_{4,228}$ = 5.01, p =0.0007, $\hat{\eta}^2_{\Lambda,r}$ = 0.06) and redilation time (MANOVA $F_{4,225}$ = 3.39, p = 0.01, $\hat{\eta}^2_{\Lambda,r}$ = 0.04) than those of the TD group. The mean PLR latency of the NDD group appeared to be longer than that of the ASD group, but the difference was not statistically significant (MANOVA $F_{4,152}$ = 1.71, p = 0.15). No significant group differences were found for other PLR parameters.

Children with ASD had a significantly higher heart rate than that of typical controls in all 5 test phases ($F_{5,218}$ = 3.32, p = 0.007, $\hat{\eta}^2_{\Lambda,r}$ = 0.05) (Table 3). The mean values of SDNN and rMSSD were lower in the ASD group than the TD group. However, MANOVA revealed that these differences were not statistically significant ($F_{5,217}$ = 2.00, p = 0.08 and $F_{5,217}$ = 1.46, p = 0.20 for SDNN and rMSSD, respectively). The AHR of the NDD group was significantly higher than that of the ASD group (MANOVA $F_{5,146}$ = 2.63, p = 0.03, $\hat{\eta}^2_{\Lambda,r}$ = 0.05). The NDD group also had a significantly higher AHR ($F_{5,132}$ = 5.41, p = 0.0001, $\hat{\eta}^2_{\Lambda,r}$ = 0.14), lower SDNN ($F_{5,131}$ = 4.70, p = 0.0006, $\hat{\eta}^2_{\Lambda,r}$ = 0.12) and lower rMSSD ($F_{5,131}$ = 2.63, p = 0.03, $\hat{\eta}^2_{\Lambda,r}$ = 0.06) than those of the TD group.

In the TD group, the PLR latency decreased from 6 to 8 years and reached a plateau thereafter (Fig. 2). The lines in Fig. 2 show the best curve fitting results using an exponential decay function $y = a\exp(-bx) + c$ with the curve-fitting tool in Matlab (Mathworks, MA). The TD results were well fitted with this function, with R^2 ranging from 0.56 to 0.88. However, either the ASD results could not be fitted with this exponential decay function or the decay was much slower than the TD results. The age effect was also not significant in the NDD group, although the number of participants was much smaller.

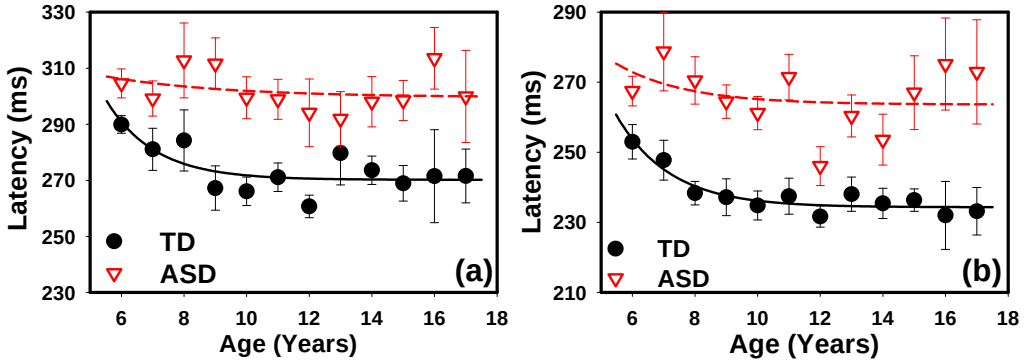

Figure 2. PLR latency vs. age measured in the TD and ASD groups at different stimulus conditions: (a) LA 69.3 cd/m^2, (b) LA 872.1 cd/m^2. The lines are fitting results using an exponential decay function y=a*exp(-b*x)+c. The error bars indicate the standard error.

A significant age effect was found on AHR and both time- and frequency-domain HRV parameters. The AHR decreased with age in both groups. SDNN showed little change before 12 years of age but was increased in older children. HF_N decreased with age in both the TD and

ASD groups. A similar age effect on AHR was observed in the NDD group, but the time domain and the frequency domain parameters did not show a significant age effect in this group.

No significant medication effect was found on PLR parameters. The ASD "w/med" group had greater AHR and lesser SDNN and rMSSD than those of the ASD "w/o med" group. The MANOVA test indicated significant group differences between the TD and ASD "w/med" group with respect to average heart rate ($F_{5,157}$ = 3.75, p = 0.003), SDNN ($F_{5,156}$ = 2.23, p = 0.006) and rMSSD ($F_{5,156}$ = 2.53, p = 0.031). However, these parameters were not significantly different between the TD and ASD "w/o med" groups or between "w/med" and "w/o med" ASD groups. Similar results between the "w/med" and "w/o med" groups were obtained in the NDD group.

No significant IQ effect was found on PLR parameters. The TD group had significantly longer latency, lesser constriction amplitude, and shorter constriction/redilation time than both the "High IQ" and "Low IQ" ASD groups.

In the ASD group, ANOVA showed that the interaction between IQ and medication had a significant effect on PLR latency ($F_{1,501}$ = 19.46, p < 0.0001) (Fig. 3). Children in the "High IQ" group did not show a difference with medication (MANOVA $F_{4,87}$ = 0.34, p = 0.85). In the "Low IQ" group, those using medication appeared to have a longer latency than those who were not using medication. However, this difference did not reach statistical significance in the MANOVA test ($F_{4,45}$ = 1.75, p = 0.16), and the t-test was significant (p = 0.03, Bonferroni corrected) only at the highest stimulus intensity of LA 8721.1 cd/m^2.

Further analysis in the "w/o med" subgroups indicated that the "High IQ" group had a similar latency to the "Low IQ" group (MANOVA $F_{4,61}$ = 0.69, p = 0.6). However, in participants who took medication (the "w/med" group), the IQ effect was significant (MANOVA $F_{4,43}$ = 3.13, p = 0.02) and children in the "Low IQ" group had a longer latency than those in the "High IQ" group.

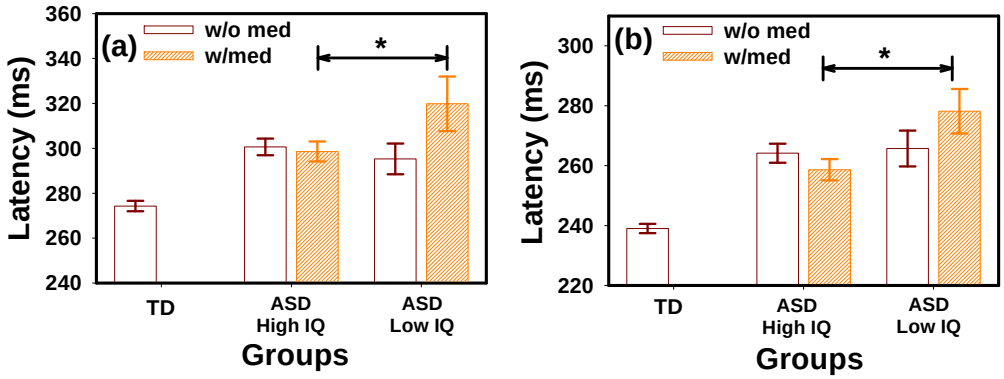

Figure 3. The effect of IQ and medication interaction on PLR latency at stimulation intensities of (a) LA69.3 cd/m^2 and (b) LA872.1 cd/m^2 in the ASD group. *MANOVA $F_{4,43}$ = 3.13, p = 0.02.

The above interaction effect was not significant on other PLR parameters or on any HRV parameters in the ASD group. In addition, the above interactions were not significant in the NDD group.

The changes of the 2 frequency domain HRV parameters between 2 adjacent test phases are shown in Fig. 4. HF_N decreased when transiting from resting phases to test phases (phase 1 to 2 and phase 3 to 4) and increased when transiting from test phases to resting

phases (phase 2 to 3 and phase 4 to 5). The changes in the LF/HF parameters were opposite of those observed in HF_N. The HF_N changes were significantly larger in the TD group than in the ASD groups (MANOVA $F_{4,226} = 4.81$, $p = 0.001$). However, the LF/HF ratio changes between the TD group and the ASD and NDD groups were not significant (MANOVA $F_{4,231} = 1.73$, $p = 0.14$). The above changes were not significant between the ASD and NDD groups (MANOVA $F_{4,157} = 0.81$, $p = 0.52$ and $F_{4,160} = 0.99$, $p = 0.42$ for HF_N changes and LF/HF ratio changes, respectively).

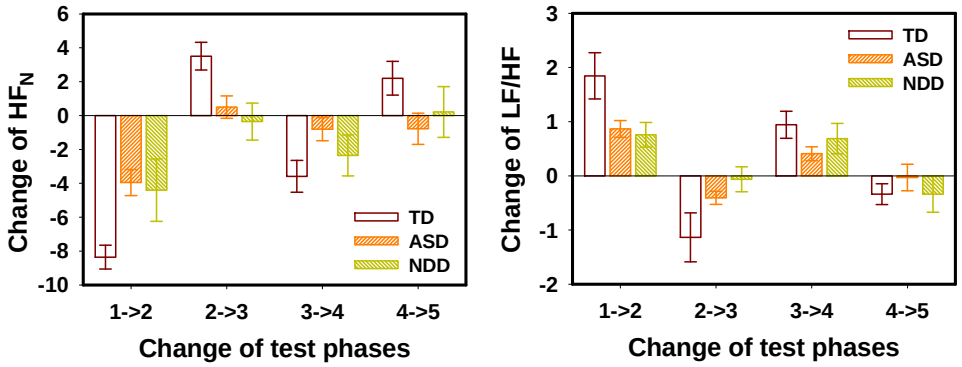

Figure 4. The change of frequency domain HRV parameters between consecutive testing phases. (a) HF normalized power and (b) LF/HF ratio. The error bars indicate the standard error. The testing phases are numbered as 1: before PLR test, 2: during LA PLR, 3: during dark adaptation, 4: during DA PLR and 5: after PLR test.

PLR constriction amplitude was significantly correlated with average heart rate in the ASD group in all LA tests ($r = -0.3$, $p < 0.01$) (Fig. 9). This correlation was observed in both the "w/o med" ASD and 'w/med" ASD groups. However, this correlation was not observed in typically developing children ($p > 0.05$). This correlation was significant in the NDD group only at the highest stimulus intensity of LA 872.1 cd/m^2. Correlations were not found between other PLR and HRV parameters.

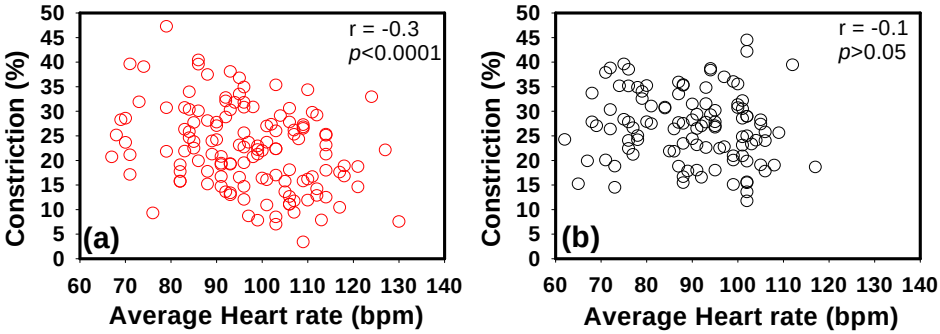

Figure 5. The correlation between average heart rate and relative constricition amplitude in (a) children with ASD and (b) typical controls. The data shown were measured at stimulus intensity of LA 872.1 cd/m^2. (Pearson's r = -0.3**, -0.3*, -0.3**, -0.1 [a] in the ASD group and r = -0.06 [a], -0.1[a], -0.1[a], -0.02[a] in the TD group at stimulus LA 69.3 cd/m^2, LA 872.1 cd/m^2, LA 8721.1 cd/m^2, and DA 63.1 cd/m^2, respectively. *p<0.0001, **p<0.01, [a] p>0.05).

Discussion

The current results confirmed the previous observation by Fan et al. (2009) that children with

an ASD had longer latency and lesser relative constriction than children with typical development. Furthermore, we found that the constriction time and redilation time were lesser in children with an ASD compared to children with typical development. Due to the predominance of male participants in this study, we also analyzed the data with only the male participants, and all group differences remained the same.

It is interesting that the age trend of PLR latency observed in typically developing children was not observed in the ASD group. This age trend in typical controls is different from the maturation of the visual system characterized by pattern visual evoked potential (VEP), which stabilizes after 6 months of life (McCulloch and Skarf 1991), but is similar to the trend observed in flash VEP (Dockstader et al. 2012). In addition, this age trend is coincident with the white matter maturation trend revealed in diffuse-tensor MRI studies (Ben Bashat et al. 2007). It has been reported that children with an ASD have accelerated white matter maturation before 4 years of age (Ben Bashat et al. 2007), but this trend is reversed after 4 years of age (Vissers et al. 2012). This appears to be consistent with our observation on PLR latency (Fig. 2).

The ASD group showed a greater average heart rate than that of the typically developing controls, which is similar to previous findings. The greater average heart rate suggests an increased sympathetic tone or/and impaired parasympathetic control in children with an ASD. The ASD group also had smaller PLR constriction amplitude, indicating lower parasympathetic modulation. Interestingly, a statistically significant negative correlation existed between PLR constriction and average heart rate in the ASD group but not in the typically developing children. The observations described above imply ANS dysfunction in children with ASD.

Frequency-domain HRV parameters appeared to be different during different test phases in both the ASD and TD groups. The PLR test requires the participant to incline slightly forward (~15°), and this posture change can cause elevation in sympathetic tone due to muscle stress. Psychological stress can also increase low-frequency HRV while decreasing high-frequency HRV. Nevertheless, the observation of significantly smaller test phase-related HRV changes in the ASD group suggested less variability in vagal and sympathetic modulation in this population. This is similar to the results reported by Toichi and Kamio (2003), who found that typical controls showed a significant decrease in cardiac autonomic function during a mental arithmetic task while the ASD group did not show significant changes. The observation in the ASD group was not caused by medication because the conclusion remained the same with only the “w/o med” ASD group used in the data analysis.

The current results did not support a significant IQ effect on PLR parameters. The apparent IQ effect on PLR latency was complicated by the medication effects. The analysis of the interaction between IQ and medication supported the notion that IQ alone does not have a significant effect on PLR latency. In the “w/o med” ASD group, where the medication effect was excluded, those in the “High IQ” group showed similar latencies as those in the “Low IQ” group. Medication effect was not observed in the “High IQ” group; however, in the “Low IQ” ASD group, latency tended to be greater in children using medication than in those not using medication. Children in the “Low IQ” group may have required medications for their severe symptoms. In other words, the observed longer PLR latency in this group of participants was associated with their symptoms rather than with medication. Similar effect of IQ and medication interaction was not observed in other PLR and HRV parameters. A trend of medication effects was observed in the results especially on average heart rate and time-

domain HRV parameters. However, the difference between "w/o med" and "w/med" ASD groups did not reach statistical significance. This observation requires further investigation.

Description of progress in Task #2:

Protocol

There are no previous studies of PLR using functional MRI techniques. As such, it was necessary to adapt the methodology used in previous PLR studies (and Task #1) for use with functional MRI methodology and MRI-safe eye tracking equipment. Data from 7 pilot participants was collected to verify that all components of the combined PLR/ fMRI paradigm provided adequate data quality. We confirmed that a rear-projection system for visual presentation of stimuli provided sufficient luminance to induce PLR in 2 participants. Additional pilot data from 5 participants was collected to verify that PLR parameters could be extracted from data provided by an MRI-compatible video eye-tracking system. Additionally, we confirmed that significant PLR-related changes in brain activity could be detected using the current combined paradigm.

In this paradigm, participants performed a passive viewing task in which they were shown a series of red-filtered, emotionally-neutral images (e.g., landscapes) that changed every 5s to maintain the interest of the participant. Every 20s, the participant was presented a green-filtered light stimulus superimposed over the current image for 100ms. The light stimulus was designed to induce PLR. For each participant, PLR and neural responses were recorded for a total of 96 light stimulus trials. Trials were present over the course of 8 functional MRI runs, each of which lasted approximately 4 ½ minutes.

MRI scans were obtained on a 3T Siemens Trio scanner with a standard 8-channel head coil. For alignment purposes, a set of structural images were collected first using a standard T1-weighted pulse sequence [MP-RAGE sequence: TR = 2400 ms, TE = 3.16 ms, flip angle = 8°, in-plane resolution = 1 x 1 mm, slice thickness = 1 mm, number of slices = 176]. For the PLR functional runs, sets of 38 contiguous axial images (TR = 2500 ms, TE = 30, flip angle = 90°, in-plane resolution = 4.0 x 4.0 mm, slice thickness = 4.0 mm) were acquired parallel to the anterior–posterior commissure plane. This procedure offered whole-brain coverage, including the cerebellum, at a high signal-to-noise ratio.

Participants

In addition to the 7 participants that were run as part of the previously described piloting of the novel PLR/fMRI paradigm, we have collected data from 25 adolescents with ASD and an additional 25 age-matched typically developing adolescents. All individuals with ASD have met diagnostic criteria on either the Autism Diagnostic Interview-Revised or the Autism Diagnostic Observation Schedule in additional to a clinical diagnosis.

Current Results

Data from 10 participants (5 ASD, 5 non-ASD) was omitted from further analysis due to excessive head motion and/or other issues (e.g., unable to complete task). [Note that this rate of data dropout (10 of 50 subjects = 20%) is very typical for MRI research with children and clinical populations.] The remaining dataset comprises of 20 participants with ASD and 20 participants without ASD. Looking forward, we anticipate completing data collection very shortly (i.e., within the 1st quarter of Year #3) and full analysis of the data will be initiated

immediately thereafter. As per the research plan, data analysis will center on detecting and localizing ASD-related differences in neural activity during PLR task performance.

KEY RESEARCH ACCOMPLISHMENTS:

- We confirmed atypical PLR in children with ASD in a large heterogeneous population;
- We found PLR latency decreased significantly from 6 to 8 years of age in typically developing children. *This age trend did not occur in children with ASD*;
- We found significant different HRV profiles in children with autism;
- Tested a total of 57 participants (7 pilot participants; 25 adolescents with ASD; and 25 typically developing adolescents without ASD) in the fMRI part of the study.

REPORTABLE OUTCOMES:

1. C. Daluwatte, J. H. Miles, S. E. Christ, D. Q. Beversdorf, N. Takahashi, G. Yao, "Atypical pupillary light reflex and heart rate variability in children with autism spectrum disorder," J. Autism and Developmental Disorders, in review (2012).
2. C. Daluwatte, J. H. Miles, S. E. Christ, D. Q. Beversdorf, A. Lofgreen, N. Birliner, G. Yao, "Age-dependent pupillary light reflex in children with autism," 34th Annual International Conference of IEEE-EMBS, San Diego, CA, Aug. 28- Sept. 1, 2012.
3. C. Daluwatte, J.H. Miles, and G. Yao, "Simultaneously measured pupillary light reflex and heart rate variability in healthy children," Physiol. Meas. 33, 1043 (2012).
4. C. Daluwatte, J. H. Miles, S. E. Christ, D. Q. Beversdorf, N. Takahashi, G. Yao, "Atypical pupillary light reflex and heart rate variability in children with autism," 2012 International Meeting For Autism Research, Toronto, Ontario, May 17-19, 2012.

CONCLUSION:

We have completed Task 1 and are on track to complete Task 2. So far into the project, we have not encountered problems in the two Tasks.

In Task 1, our results showed that the ASD group had significantly longer PLR latency, reduced relative constriction amplitude, and lesser constriction/redilation time than those of the TD group. Similar atypical PLR parameters were observed in the NDD group. A significant age effect on PLR latency was observed in children younger than 9 years in the TD group, but not in the ASD and NDD groups. Atypical HRV parameters were observed in the ASD and NDD groups. A significant negative correlation existed between the PLR constriction amplitude and average heart rate in children with an ASD, but not in children with typical development.

We will continue to work on Task #2 and complete fMRI data acquisition and analysis as proposed.

This project serves as an important step to validate and further understand the atypical PLR in autism. As a quick, non-invasive and objective test, PLR can provide quantitative measures of specific neurologic aspects of autism and thereby facilitate our understanding of this complex disorder.

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Atypical pupillary light reflex and heart rate variability in children with autism

Chathuri Daluwatte[1], Judith H. Miles[2], Shawn E. Christ[3], David Q. Beversdorf[2,3,4,5], Nicole Takahashi[2], Gang Yao[1]
[1]Department of Biological Engineering,
[2]Thompson Center for Autism & Neurodevelopment Disorders,
[3]Department of Psychological Sciences,[4]Department of Radiology, [5]Department of Neurology,
University of Missouri, Columbia, MO, USA.

Background: Atypical pupillary light reflexes (PLR) were previously reported in children with Autism Spectrum Disorder (ASD). A replication study is being conducted in a larger population to further investigate PLR profiles in children with ASD. Heart rate variability (HRV) was also measured simultaneously to explore potential impairments in the autonomic nervous system (ANS) associated with ASD.

Objectives: To study PLR and HRV profiles in children with ASD.

Methods: PLR and HRV were analyzed in 143 children with ASD (age 10.7±3.4 years, 128 males and 15 females) and 109 children of typical development (age 11.0±2.9 years, 80 males and 29 females). PLR induced by a 100ms green light was measured in both light adapted (LA) and dark adapted (DA) conditions using a two channel binocular apparatus. Five basic PLR measurements including resting pupil diameter, relative constriction, latency, constriction velocity and redilation velocity were calculated to quantify PLR. HRV was measured using a remote heart rate device during the entire PLR test. In addition to time domain HRV parameters, Fourier transform was applied to calculate the high frequency ("HF") and low frequency ("LF") components of the RR tachogram power spectrum.

Results: Similar to the previous findings, children with an ASD had significantly longer PLR latency ($p < 0.0001$) and smaller PLR constriction ($p = 0.0034$) than the typical controls. In typical controls, the PLR latency decreased significantly from 6 to 8 years old (one way ANOVA $p < 0.05$) and stabilized thereafter. No significant age effect was observed in latency obtained in the ASD group. The average heart rate was significantly higher in children with an ASD ($p < 0.05$). The control group showed lower normalized HF power (high frequency power divided by total of high frequency and low frequency power) and higher LF/HF ratios (ratio between high frequency power and low frequency power) during the PLR test than during the resting periods ($p < 0.05$). The same change was also observed in the ASD group, but the magnitude of change was much smaller than that of the controls.

Conclusions: The atypical PLR profiles found in our preliminary study were confirmed in a larger ASD population in this study. The different age effect on PLR latency suggests that the developmental trajectory associated with PLR pathway may be altered in children with ASD. The observed high average heart rate indicated elevated sympathetic tone in the ASD group. HRV changes during administration of the PLR (higher LF/HF and lower HF power) suggest that children with ASD have an altered ANS response to the PLR.

Yao, Gang (Gary)

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Sent: Thursday, July 05, 2012 12:33 PM
To: Daluwatte, Chathuri L. (MU-Student)
Cc: Miles, Judith H.; Christ, Shawn E.; Beversdorf, David; agld4f@mail.missouri.edu; Berliner, Nathan K. (MU-Student); Yao, Gang (Gary)
Subject: Decision on EMBC'12 submission 216
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Chathuri Lakshika Daluwatte*, Judith Miles, Shawn Christ, David Beversdorf, Andrew Lofgreen, Nathan Berliner, Gang Yao Age-Dependent Pupillary Light Reflex Parameters in Children

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Ms. Jessica Lotito

EMBS
IEEE
445 Hoes Lane
Piscataway, NJ 08854
United States of America
E-mail address: j.lotito@ieee.org

Age-dependent pupillary light reflex parameters in children*

Chathuri Daluwatte, Judith H. Miles, Shawn E. Christ, David Q. Beversdorf,

Andrew Lofgreen, Nathan Berliner, Gang Yao, *Member, IEEE*

***Abstract*— Pupillary light reflex (PLR) refers to the phenomenon where pupil size changes in response to stimulation with a flash of light. It is a simple functional test that can reveal dysfunctions associated with the PLR pathway. Although abnormal PLR responses have been reported in many neurological disorders, few studies investigated neurodevelopmental effects on PLR parameters. We studied the effect of age on PLR in a group of 6 to 17 year old children with typical development. A significant and consistent age effect was found on PLR latency in children younger than 10 years old. Age effects were also observed in resting pupil diameter and constriction amplitude. However such age related trends were not observed in children with neurodevelopment disorders. These results suggest that PLR has the potential to be used as a simple noninvasive tool for monitoring neurodevelopment in children.**

I. Introduction

American Academy of Pediatrics (AAP) estimates that 12% to 16% children have some forms of developmental disorders [1]. Substantial clinical evidence supports that early intervention leads to improved functioning. Early detection is essential to ensure early intervention [1]. In the United States developmental screening is presumed be done in the pediatrician's or family doctor's office using one or more screening questionnaires [2]. Unfortunately, this practice is neither consistent nor universal which leads to considerable lag in the diagnosis for children with developmental disabilities [1]. In addition, behavioral symptoms usually lag behind the underlying neurophysiological changes. Therefore there is a need for an objective measure that can accurately track normal neurodevelopment progress in children.

Pupillary light reflex (PLR) is tested by measuring pupil size change in response to a short light flash. The size of the pupil is controlled by two antagonistic iris muscles: the sphincter and the dilator that are innervated by different neurological systems [3]. Photoreceptors in the retina detect and convey the sensory information about retinal illumination to the pretectal olivary nucleus (PON) via optic nerves. The PON synapses at the Edinger Westphal (EW) nucleus [4] which then projects to the ciliary ganglion to control the sphincter muscle via the short ciliary nerve [5, 6]. The neurological pathway related to pupil dilation is still not well understood [5]. The dilator muscle receives control from the superior cervical ganglion via the ciliary nerves. The ciliospinal center of Budge is found to project to the superior cervical ganglion [7].

PLR responses can be altered by dysfunctions in the PLR pathway. In fact, abnormal PLRs have been previously reported in several types of neurological disorders. Fan el al. [8] reported prolonged PLR latency, smaller relative constriction and lower constriction velocity related to autism spectrum disorder (ASD). Giza el al. [9] reported prolonged latency, reduced amplitude, maximum constriction velocity and maximum acceleration associated with Parkinson's disease. Fotiou et al. [10] reported atypical PLR associated with Alzheimer's disease, where all parameters except baseline and minimum pupil diameters were affected.

To develop an effective screen for neurodevelopment disorders, it is important to first understand neurodevelopment in typically developing children. Several studies have been conducted to examine the normal neurodevelopmental progress of the visual system in children by using visual evoked potentials (VEP) [11, 12]. A recent report demonstrated the potential of using PLR to examine visual system development in preterm babies [13]. However, no comprehensive study has been conducted to investigate age related profiles of PLR parameters in children.

Here we report our results of PLR tests in over 100 typically developing children from 6 to 17 years old. Our results revealed a significant age effect in PLR parameters, particularly the PLR latency and resting pupil diameter. A similar trend was not observed in a group of age-match children with neurodevelopmental disorders.

II. Procedure

A. Instrumentation

A custom-built binocular pupilogram recording system (Fig. 1) was used to measure PLR with high spatial (35μm/pixel) and temporal resolution (8.7 ms). The two recording channels are independent but synchronized. The optical stimulation and image acquisition were controlled through a computer interface via a custom-developed Labview program. This customized system has two "sighting" ports so that the participant can fix sight at a given target during PLR test. In addition, this system is versatile for

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C. Daluwatte is with Department of Biological Engineering, University of Missouri, Columbia, MO 65211, USA. (phone: 573-882-3454, e-mail:cldc82@mail.missouri.edu)

J. H. Miles is with Thompson Center for Autism & Neurodevelopment Disorders, University of Missouri, Columbia, MO 65201, USA. (e-mail: MilesJH@missouri.edu)

S. E. Christ is with Department of Psychological Sciences, University of Missouri, Columbia, MO 65211, USA. (e-mail: ChristSE@missouri.edu)

D. Q. Beversdorf is with Department of Radiology, Department of Neurology, Department of Psychological Sciences, and Thompson Center for Autism & Neurodevelopment Disorders, University of Missouri, Columbia, MO 65201, USA. (e-mail: BeversdorfD@missouri.edu)

A. Lofgreen, N. Berliner and G. Yao are with Department of Biological Engineering, University of Missouri, Columbia 65211, MO, USA (e-mail: YaoG@missouri.edu).

setting various stimulation waveforms and intensities.

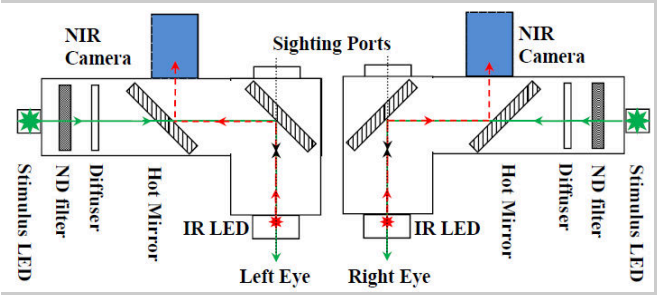

Figure 1. A schematic diagram of the binocular pupilogram recording system. A hot mirror was used in each channel to separate the optical stimulation path and imaging path. The participant can fix the sight on a monitor through the two sighting ports.

Pupils were illuminated by near infrared (NIR) LEDs at 880 nm wavelength. A 530 nm green LED was used to provide the light stimulus for evoking the PLR. The electric current to the LED was controlled to vary the stimulation irradiance along with the use of neutral density (ND) filters. The stimulation light then passed through a diffuser providing on-axis illumination with 5.7° visual field. The stimulation intensities used in this study varied from 0.09 $\mu W/cm^2$ to 9.9 $\mu W/cm^2$ in light-adaptation (LA) and was 0.09 $\mu W/cm^2$ in dark-adaptation (DA).

Two near infrared (NIR) cameras (GC660, Allied Vision Technologies, Stadtroda, Germany) were used in the system to acquire pupil images. The image size was 659 pixels × 494 pixels with a 12 bit resolution. At each PLR test, the cameras were triggered first to acquire baseline pupil images for 1s. Then the green LEDs were triggered to give a 100ms flash. Image acquisition was continued for four more seconds to capture the entire pupil constriction and recovery process. A total of 575 images were acquired from each eye in a single test trial (5 sec). All acquired images were saved using the tiff format.

Custom image processing software developed in visual c++ was used to automatically calculate the pupil diameter from each of the recorded pupil images in the image sequence (575 images for each eye). A histogram-based threshold method was applied after contrast stretching the pupil image to locate boundary pixels for the pupil. The threshold of pupil boundary was identified as the pixel value corresponding to first minima of the image histogram as shown in Fig. 2(b). Using this threshold the images were binarized and pupil was segmented. All pixels on the pupil boundary were then extracted. An ellipse was fitted to the segmented pupil boundary (Fig. 2(a)) by using a direct least square fitting algorithm [14]. The area of the fitted ellipse was used to estimate the pupil area. A nominal diameter was calculated by treating the pupil as a circle.

Once all pupil diameters were extracted from the acquired image sequence, a pupilogram curve (Fig. 3) was constructed to represent the pupil size change in response to the optical stimulus. The pupilogram was normalized against the resting pupil area to remove effects of resting pupil size when calculating constriction amplitude. The following PLR parameters were calculated from the pupilogram in Fig. 3 to quantify the pupillary response. The resting pupil diameter D_0 was calculated by averaging pupil diameters obtained during the 1s period before stimulus onset.

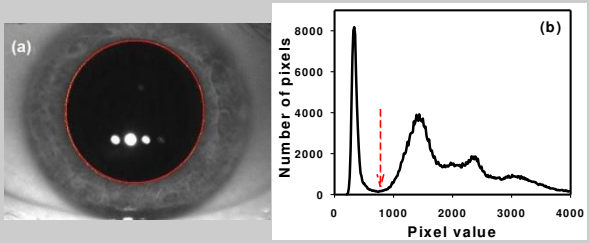

Figure 2. An example to illustrate the pupil segmentation used in our study. (a) An example pupil image. (b)The corresponding histogram. The first minima marked by the arrow in (b) indicates the boundary of the black pupil in (a). This value was used as the threshold to segment the pupil. The red circle in (a) shows the fitted ellipse using least square fitting.

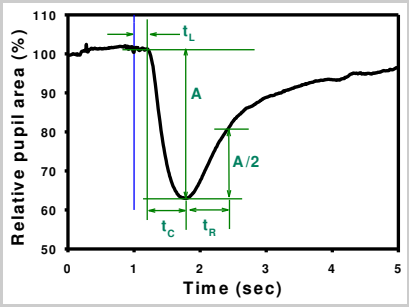

Figure 3. An illustration of the pupilogram which is normalized against the pupil area before stimulus onset (the resting pupil area D_0^2). Following extracted PLR parameters are shown: A= relative constriction amplitude; t_L = latency; t_C = constriction time; t_R= redilation time

The relative constriction amplitude was calculated by normalizing the difference between resting pupil area and minimum pupil area against the resting pupil area. PLR latency (t_L) was calculated as the time interval between stimulus onset and the beginning of pupil constriction. The constriction time (t_C) was calculated as the time interval between the beginning of pupil constriction and when pupil reached minimal size. The redilation time (t_R) was calculated as the time interval between the minimal pupil diameter and when the pupil recovered to half of the constriction. The pupilogram (before normalization) was smoothed by using a 6^{th} order Savitzky-Golay filter. To measure the PLR latency, the acceleration (2^{nd} order derivative) of the pupilogram was calculated. The time of the maximal acceleration was first identified and used as the starting point to back-track toward the stimulation onset. The first image frame that deviated from the baseline pupil size was considered as the onset of pupil constriction.

B. Test procedure

PLR data were obtained in 107 healthy children 6 to 17 years old (mean age 10.9±2.9 years) without any known visual or neurological problems. There were 79 males (mean age 10.9±3.1 years) and 28 females (mean age 10.6±2.4 years). As a comparison, PLR data were also examined in 176 children (mean age 10.5±3.1 years, 150 males and 26 females) with several different types of neurodevelopmental disorders including autism (147), mental retardation or developmental delays (10), Down's syndrome (7), Fragile

X syndrome (5), cognitive disorders (4) , learning disability (1), Prader Willi (1), Oppositional defiant disorder (1). This group of participants was recruited through the Thompson Center for Autism and Neurodevelopmental Disorders at University of Missouri. Written consents were obtained from all participants and their legal guardians as approved by the Institutional Review Board of University of Missouri-Columbia.

PLR was measured in both light adapted (LA) (room luminance of 30cd/m^2) and dark adapted (DA) (<0.02cd/m^2 room luminance) conditions. The intensities used as optical stimulation for PLR were 0.09μW/cm^2 in dark-adaptation, and 0.09μW/cm^2, 1.0μW/cm^2, 9.9μW/cm^2 in light adaptation. For each stimulus condition, PLR responses from both eyes were measured when one eye was stimulated. The measurements were repeated four times for each condition with an approximately 30s interval between two consecutive measurements. Imaging was started 1s before the stimulation to gather the resting pupil size. After the LA test, all participants stayed in the dark room for 15 minutes for the pupils to naturally dilate before starting the DA test.

C. *Data analysis*

The Analysis of Covaraince (ANCOVA) was applied in SAS to examine the effects of age and test conditions on each PLR parameter. Follow up analysis of variance (ANOVA) was performed to verify the age effect for a linear relationship. PLR parameters were verified for normal distribution using the Kolmogorov-Smirnov test. p<0.05 was considered as significant.

III. Results

As expected, in typically developing children the resting pupil diameter was larger in dark adaptation (7.44±0.77 mm) than in light adaptation (6.58±0.61 mm) as shown in Fig. 4. The resting pupil diameter increased with age significantly before 12 years old (F(6,135) = 2.67, p=0.018). From 6 to 12 years old, the mean resting pupil diameter increased 8.0% in LA and 13.2% in DA. The ANOVA test for a linear trend further confirmed that the age effect was significant (p=0.047 at LA and p= 0.003 at DA). At the same stimulus intensity, the PLR constriction amplitude was larger in dark-adaption whereas the constriction/redilation times were longer and latency was shorter. In light-adapted tests, as stimulus intensity increased from 0.09μW/cm^2 to 9.9μW/cm^2, PLR latencies decreased 21.85%; constriction and redilation times increased 25.30% and 48.15% respectively; and relative constriction amplitude increased from 11.76±5.54% to 40.75±7.23%.

The ANCOVA model suggested a significant age effect on several PLR parameters. In children from 6 to 8 years old, the age effect was significant for constriction amplitude (F(3,132)=3.48, p=0.018). PLR constriction increased with age in children younger than 8 years old and reached a plateau thereafter (Fig. 5a) at all stimulation conditions except the one at LA 0.09μW/cm^2. However the linear increasing trend at young age (< 8 years) was significant only with the maximal stimulus at LA 9.9μW/cm2 (F(1,21)=5.70, p=0.027). The PLR constriction time and the redilation time did not show an effect with age.

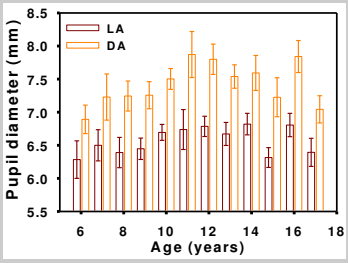

Figure 4. The age effect in resting pupil diameter in the light adapted (LA), and dark adapted (DA) environment in children with typical development. The error bars indicate the standard error.

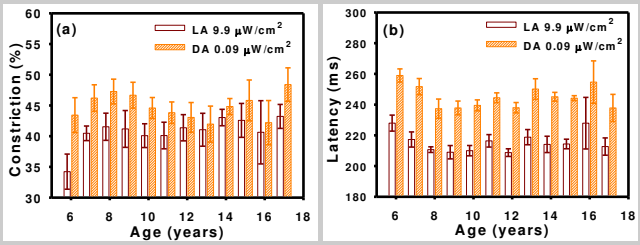

Figure 5. PLR parameters obtained in children with typical development from 6 to 17 years old. (a) Relative constriction amplitude, (b) latency measured in the light adapted (LA) 9.9μW/cm^2 and dark adapted (DA) 0.09μW/cm^2 condition. The error bars indicate the standard error.

The most consistent age effect was observed in PLR latency. The ANCOVA model revealed that in children from 6 to 9 years old, latency has a significant age effect (F(3,132) = 6.68, p<0.001). As shown in Fig. 5b, PLR latency decreased significantly at all testing conditions from 6 to 9 years and stabilized thereafter. For example, the PLR latency decreased from 259.0±4.3ms at 6 years old to 237.3±6.3ms at 8 years old at stimulation condition of LA 9.9μW/cm^2. The ANOVA test for a linear trend in children younger than 10 years old further confirmed that the age effect was significant (p< 0.01) at all conditions except the one with the lowest stimulus intensity of LA 0.09μW/cm^2.

Since we saw a consistently significant age trend in PLR latency, we examined the age trend in PLR latency and resting pupil diameter measured in a group of children of the same age range with neurodevelopment disorders. As shown in Fig. 6, no age dependent trend in PLR latency existed in this group of children. At the same stimulation condition, children with neurodevelopment disorders had significantly longer latency than typically developing children. Similarly we didn't observe any age effects on resting pupil diameter in this group of children.

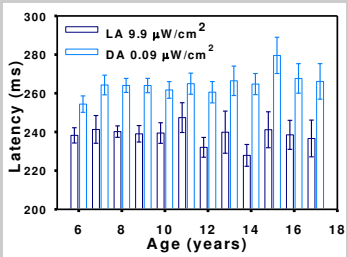

Figure 6. The PLR latency in children with neurodevelopment disorders measured at light adapted (LA) 9.9 μW/cm^2 and dark adapted (DA) 0.09μW/cm^2 condition. The error bars indicate the standard error.

IV. Discussion

PLR is an involuntary neurological response. Testing of PLR is noninvasive, simple and fast. It requires minimal cooperation from the subject and thus is convenient for testing in children. A good understanding of age dependent behavior of PLR is essential to evaluate the potential use of PLR for screening neurodevelopmental disorders in children.

Our results indicated a consistent and statistically significant age effect in PLR latency measured in young children (<10 years old) with typical development. These results appear to be consistent with previous findings of age-dependent changes in visual evoked potential (VEP) in children. Lenassi el al. [12] compared flash VEP and pattern VEP in infants and young children from 1.5 months to 7.5 years of age. They found that VEP latency for all three stimulation types showed an exponential decrease with age, but the trends were different. The latencies of reversal and pattern onset VEP showed fast decays (exponential decay rate of -9.3/year and -13/year respectively) and were stabilized by 6 months of age. However, flash VEP latency showed a slower decay (exponential decay rate of -0.54/year) and still decreased gradually at the upper limit of the age (7.5 years) they tested. Our age-dependent PLR latency in children (6 – 9 years) with typical development had a similar effect as the flash VEP latency results reported by Lenassi el al. [12]. Carrillo-De-La-Pena el al. [11] studied flash VEP in 85 children from 8 -15 years old and reported no significant age effect in latency. This result is consistent with our observation that PLR latency didn't change in children older than 9 years old.

Although a significant age effect was reported in relative constriction, it was statistically significant only at one test condition with the highest stimulus intensity. With a close examination, we noticed that the coefficient of variance for relative constrictions varied from 12% to 63% at those stimulation conditions where the age effect was not statistically significant. At the strongest stimulus of LA at 9.9 $\mu W/cm^2$, the coefficient of variance was much smaller, from 8% to 24%. Hence it is possible that the lack of statistical significance can be attributed to the higher variation in data obtained with smaller stimulus intensities.

The fact that no age-dependent trend in PLR latency or resting pupil diameter was observed in the group of children with neurodevelopment disorders suggests that the typical neurodevelopmental trajectory might be altered in neurodevelopmental disorders. The underlying mechanisms need further study. However, our result suggests that PLR has the potential to provide clinically useful information about progression of neural development in children.

V. Conclusion

We found a significant and consistent age dependent effect in PLR latency in children 6 to 9 years old. We also observed age effects in resting pupil diameter and PLR constriction amplitude. Such an age-dependent effect was not observed in children with neurodevelopment disorders. Further studies in larger groups of children especially in children younger than 6 years old are necessary to fully understand the details of age dependency of PLR. Nevertheless, PLR shows potential to be applied as a simple noninvasive tool to monitor neurodevelopment in children.

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Simultaneously measured pupillary light reflex and heart rate variability in healthy children

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Simultaneously measured pupillary light reflex and heart rate variability in healthy children

C Daluwatte[1], **J H Miles**[2] **and G Yao**[1]

[1] Department of Biological Engineering, University of Missouri, Columbia, MO 65211, USA
[2] Child Health and Thompson Center for Autism and Neurodevelopmental Disorders, University of Missouri, Columbia, MO 65211, USA

E-mail: YaoG@missouri.edu.

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Abstract

We investigated the potential inter-relationship between two measures of autonomic nervous system: pupillary light reflex (PLR) and heart rate variability (HRV), in healthy children of 8–16 years old. PLR was measured at both dark- and light-adapted conditions with various stimulation intensities. Simultaneously measured HRV was obtained in five different PLR testing phases: before PLR test, light-adapted PLR test, dark adaptation, dark-adapted PLR test and after PLR test. The frequency domain HRV parameters measured during the PLR test were significantly different from those measured during rest. Both the regression analysis and factor analysis indicated that PLR and HRV parameters were not correlated, which suggests that they may provide complementary assessment of different aspects of the overall autonomic nervous system.

Keywords: pupillary light reflex, heart rate variability, children

(Some figures may appear in colour only in the online journal)

1. Introduction

The autonomic nervous system (ANS) is a complex and pervasive system that controls the critical visceral functions of the human body and is involved in many psychophysiological responses. Its two divisions, the sympathetic and parasympathetic systems, act in a complementary manner regulated by the central autonomic network (Levy 1997). In such a highly integrated organization, ANS dysfunctions are often widespread with symptoms appearing in multiple subsystems. Hence, an interesting question arises: whether an assessment of a specific ANS subsystem may reflect the overall physiological status of the system, and how variations in different measures of ANS correlate with each other (Bär *et al* 2009). This study investigated the possible inter-relationship between two specific ANS measures: pupillary light reflex (PLR) and heart rate variability (HRV).

PLR refers to the change of pupil size in response to luminance changes. The pupil size is controlled by two iris muscles, the sphincter and dilator, which produce pupil constriction and dilation, respectively. The sphincter is mainly innervated by the parasympathetic nervous system (PNS), whereas the dilator is innervated by the sympathetic nervous system (SNS) (Barbur 2004). The parasympathetic nerve fibers synapse in the ciliary ganglion and control the sphincter muscle via the short ciliary nerve (Lowenstein and Loewenfeld 1950). These fibers originate from the pupilloconstrictor neurons at the Edinger–Westphal (EW) nucleus that receives input from the olivary pretectal nucleus (OPN) in the midbrain. The dilator muscles are controlled by post-ganglionic sympathetic fibers from superior cervical ganglion that receives input from the ciliospinal center of Budge (Appenzeller 1999). The pupil size is determined by the balance between the sympathetic and parasympathetic systems (Fotiou *et al* 2000). Abnormal PLR has been observed in many neurological disorders associated with ANS dysfunction (Bremner 2009) such as panic disorder (Kojima *et al* 2004), autism (Fan *et al* 2009b) and Parkinson's disease (Giza *et al* 2011, Stergiou *et al* 2009).

HRV assesses the beat-to-beat variations of the heart rate. Both parasympathetic and sympathetic systems are involved in cardiovascular system regulation. Stimulation of the parasympathetic fibers (vagus nerves) reduces the heart rate, whereas the sympathetic stimulation increases the heart rate through the sinoatrial (SA) node. HRV parameters have been widely applied to evaluate cardiac autonomic functions (Kamath and Fallen 1993) and have been useful in identifying the ANS function in various allostatic systems (Thayer and Sternberg 2006). HRV has also been applied in evaluating the ANS dysfunction in disorders such as panic disorder (Yeragani *et al* 1993), schizophrenia (Bär *et al* 2005, Bär *et al* 2007) and sleep disorders (Bonnet and Arand 1998).

The possible association between PLR and HRV has recently been investigated by several authors. Correlations between HRV and PLR parameters were found in adults during exercise (Kaltsatou *et al* 2011) and in patients with acute schizophrenia (Bär *et al* 2008). However, Bär *et al* (2009) found limited correlation between specific PLR and HRV parameters in healthy adults 19–64 years old. It is unclear whether similar relationships may exist in younger subjects as both HRV and PLR are affected by age. Here we report a study that measured simultaneously the PLR and HRV in healthy 8–16 year-old children to further investigate the possible association between measures obtained from pupillary light reflex and heart rate variability (HRV).

2. Methods

2.1. Participants

A total of 54 healthy 8–16 year-old children participated in this study including 27 boys (138.2 $\pm$ 28.2 months) and 27 girls (age 136.1 $\pm$ 28.1 months) without any known vision, neurological and cardiovascular problems. All were tested at least 1 h after their last meal. Among the participants, 23 children (9 girls and 14 boys) were tested in the morning (8 am–12 pm) and the remaining 31 (18 girls and 13 boys) were tested in the afternoon (12 pm–5 pm). All participants and their legal guardians were thoroughly informed of the procedure and consented with a written informed consent as approved by the Institutional Review Board of the University of Missouri.

2.2. Instrument

A binocular pupilogram recording system was used to measure the PLR. This system is similar to that reported previously (Fan *et al* 2009a, 2009b) except that faster cameras were used to

Figure 1. An illustration of the test procedure used in this study.

record pupil images at 115 fps. The spatial resolution of the system was 35 μm/pixel. A 530 nm green LED provided the light stimulus. The stimulus pulse width was 100 ms. Neutral density filters and LED current were used to control the stimulation intensity.

To obtain HRV, real-time QRS intervals were recorded by using a remote heart rate measuring device (Polar RS800CX, Polar Electro Oy, Finland). A chest strap with enclosed heart rate sensor and wireless transmitter was wrapped around the participant's chest. The system acquires the ECG at 1 kHz rate. The heart beat QRS signals transmitted from the chest strap were received and recorded by a watch-like device. Multiple studies (Gamelin *et al* 2006, Gamelin *et al* 2008, Goodie *et al* 2000, Nunan *et al* 2009, Porto and Junqueira Jr 2009) have shown this device to be reliable for the short-term R–R interval measurement and to provide results consistent with traditional ECG.

2.3. Test procedure

Heart rate recording was started 5 min before the PLR test to acquire a baseline reference. The participant remained in a sitting position during the entire test. PLR was first measured in a light-adapted (LA) condition (30 cd m^{-2} room illumination). Three optical stimulation intensities were used in LA tests: 69.3, 872.1 and 8721.1 cd m^{-2} to induce different amounts of pupillary constriction. Following a 15 min dark adaptation (<0.02 cd m^{-2} room illumination), dark-adapted (DA) PLR was then measured at a stimulation intensity of 63.1 cd m^{-2}. At each test condition, the left eye was stimulated first and the right eye was stimulated next. Images of both pupils were recorded for the analysis. The measurements were repeated four times for each condition with a 20 s interval between measurements. Pupil imaging was started 1 s before the onset of the 100 ms optical stimulation to obtain baseline pupil size, and was recorded continuously for 4.5 s. Heart rate was continuously recorded during the PLR test and stopped 5 min after completing the PLR test. The entire test procedure is illustrated in figure 1.

2.4. Data analysis

The pupilogram was constructed by extracting the pupil size from acquired pupil images as described in detail previously (Fan *et al* 2009a, 2009b). As in our previous studies, the PLR parameters listed in table 1 were measured in this study to characterize PLR responses. PLR parameters obtained from both eyes during the four repeat measurements were averaged to calculate the mean value at any given condition.

HRV was assessed using both time domain and frequency domain methods (Malik *et al* 1996). As shown in table 2, two time domain parameters were calculated: SDNN and rMSSD. To be consistent with the most recent studies on HRV, two frequency domain parameters were analyzed: the normalized HF and the LF/HF ratio. The frequency domain power spectrum was analyzed by using fast Fourier transform (FFT) as described by Malik *et al* (1996). To study the potential effect of the PLR tests on HRV parameters, HRV was analyzed in five

Table 1. PLR parameters measured in this study.

Symbol (unit)	Definition
D_0 (mm)	'Base pupil diameter': pupil diameter before stimulus onset
$D_{\min}$ (mm)	'Minimal pupil diameter': pupil diameter before stimulus onset
$\Delta A_{\%}$	'Relative constriction amplitude': $\left(D_0^2 - D_{\min}^2\right)/D_0^2$
t_L (ms)	'Constriction latency': the elapsed time between light stimulus and beginning of constriction
t_C (ms)	'Constriction time': the time interval from the beginning of constriction to the maximal constriction
t_R (ms)	'Redilation time': the time interval between maximal constriction and recovery to half of the constriction $\left(D_0^2 - D_{\min}^2\right)/2$
v_C (mm s^{-1})	'Constriction velocity': average velocity of the relative constriction $(D_0 - D_{\min})/2t_C$
v_R (mm s^{-1})	'Recovery velocity': average velocity of the relative recovery $(D_0 - D_{\min})/(4t_R)$

Table 2. HRV parameters measured in this study.

Symbol (unit)	Definition
HR (bpm)	Average heart rate
SDNN (ms)	Standard deviation of normal RR intervals
rMSSD (ms)	Root mean square successive difference of the RR intervals
HF_n	Normalized powers of HF band (HF, 0.15–0.4 Hz), i.e. the relative powers of HF band when removing very low frequency (VLF, 0–0.04 Hz) band power from total power. HF power/(total power–VLF power) $\times$ 100%
LF/HF	The ratio of LF/HF power (LF, 0.04–0.15 Hz)

testing phases: before PLR test (5 min), LA test (10 min), dark adaptation (15 min), DA test (5 min) and after PLR test (5 min).

The distributions of PLR and HRV parameters were verified to conform to normal distributions using the Kolmogorov–Smirnov test. For each PLR and HRV parameter, the analysis of covariance (ANCOVA) using the PROC MIXED procedure in SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA) was applied to examine the effects of test conditions, gender and age. *P*-values were Bonferroni-corrected appropriately using the number of stimulus conditions for PLR and the number of testing phases for HRV. To study the association between PLR and HRV parameters, linear correlations between them were first analyzed with Pearson product moment correlation (PROC CORR procedure in SAS). To investigate whether variations in PLR parameters can be explained by a combination of HRV parameters or vice versa, multilinear regression (PROC REG procedure in SAS) was applied with HRV (or PLR) parameters treated as dependent variables and PLR (or HRV) parameters treated as independent variables. Moreover, the explanatory factor analysis (PROC FACTOR procedure in SAS) was performed on the overall data set to study the potential relationships between PLR and HRV parameters using oblique matrix rotation. The factor analysis reduces a multivariate system to a system with a fewer number of dimensions by categorizing highly correlated dependent variables into a single factor.

3. Results

3.1. Pupillary light reflex

Table 3 shows the mean values and standard deviations of all measured PLR parameters. The initial pupil diameter D_0 increased from 6.6 $\pm$ 0.6 mm in LA to 7.5 $\pm$ 0.8 mm in DA. The

Table 3. Summary of PLR results. The results are represented as group mean ± standard deviation.

	Stimulation intensity (cd m^{-2})			
	LA 69.3	LA 872.1	LA 8721.1	DA 63.1
Resting pupil diameter (mm)		6.6 ± 0.6		7.5 ± 0.8
Relative constriction amplitude (%)	12.8 ± 6.0	27.0 ± 7.4	40.8 ± 7.89	44.6 ± 7.3
Latency (ms)	269.3 ± 26.6	236.1 ± 15.8	211.8 ± 13.5	239.3 ± 15.9
Constriction time (ms)	368.6 ± 71.8	398.5 ± 56.6	463.4 ± 52.8	575.6 ± 52.3
Redilation time (ms)	417.6 ± 74.7	501.7 ± 109.9	610.7 ± 135.8	817.8 ± 172.0
Constriction velocity (mm s^{-1})	1.0 ± 0.5	2.0 ± 0.7	2.8 ± 0.8	2.8 ± 0.8
Redilation velocity (mm s^{-1})	0.5 ± 0.2	0.8 ± 0.3	1.1 ± 0.3	1.0 ± 0.0

Table 4. Summary of HRV results. The results are represented as group mean ± standard deviation.

	Testing phase				
	Before PLR test	During LA PLR test	During DA period	During DA PLR test	After PLR test
Heart rate (bpm)	89.9 ± 12.1	90.1 ± 12.1	92.7 ± 12.1	91.0 ± 13.2	93.5 ± 13.2
SDNN (ms)	67.6 ± 27.0	70.2 ± 23.9	67.9 ± 29.1	71.5 ± 28.5	65.2 ± 25.0
rMSSD (ms)	38.9 ± 20.3	36.7 ± 16.9	34.1 ± 17.7	36.4 ± 18.6	33.2 ± 16.9
HF_n (%)	31.8 ± 11.0	23.1 ± 7.1	26.9 ± 10.1	22.3 ± 8.2	24.9 ± 10.1
LF/HF ratio	2.6 ± 1.4	3.8 ± 1.6	3.2 ± 1.4	4.1 ± 2.1	3.7 ± 2.3

relative constriction amplitude $\Delta A_{\%}$ increased from 12.8% ± 6.0% at LA 69.3 cd m^{-2} to 40.6% ± 7.3% at LA 8721.1 cd m^{-2}. The PLR latency was between 200 and 300 ms. In LA tests, PLR latency decreased with stimulus intensity (F-value = 236.0, $p < 0.0001$ in ANOVA test for a linear trend). $\Delta A_{\%}$ was similar at LA 8721.1 cd m^{-2} and DA 63.1 cd m^{-2}, but the DA latency was ∼30 ms longer. Average constriction velocity v_C ranged from 1.0 to 2.8 mm‘ s^{-1} at the four stimulation conditions, whereas the average redilation velocity v_R was 0.5–1.0 mm s^{-1}.

3.2. Heart rate variability

The HRV parameters are shown in table 4. The average heart rate was around ∼ 90 bpm in our study population. The time domain HRV parameter SDNN was ∼65–72 ms on average and the rMSSD ranged between 33 and 39 ms. The frequency domain HRV parameters were HF normalized power (∼22–32) and LF/HF ratio (∼2–4).

3.3. Inter-relationship between PLR and HRV

As shown in table 4, the average heart rate, SDNN and rMSSD measured during different PLR testing phases were not significantly different (ANCOVA F-value = 1.06, 0.52 and 0.86, $p = 0.38$, 0.72 and 0.48, respectively). In fact, the HRV parameters obtained during different testing phases were highly correlated with each other. The heart rate had the highest correlation coefficient among all parameters. Table 5 shows that the correlation weakened as the measurement phase became distinct in time, i.e. the r-value decreased from 0.95 between phases 1 and 2 (5 min interval) to 0.88 between phases 1 and 5 (25 min interval).

However, the correlation of frequency domain HRV among different testing phases was not as high as the time domain parameters. The LF/HF ratio had the least correlation r-values (0.54–0.87) among different testing phases. In fact, the ANCOVA model indicated that the PLR testing phase had a significant fixed effect on the LF/HF ratio and HF normalized power

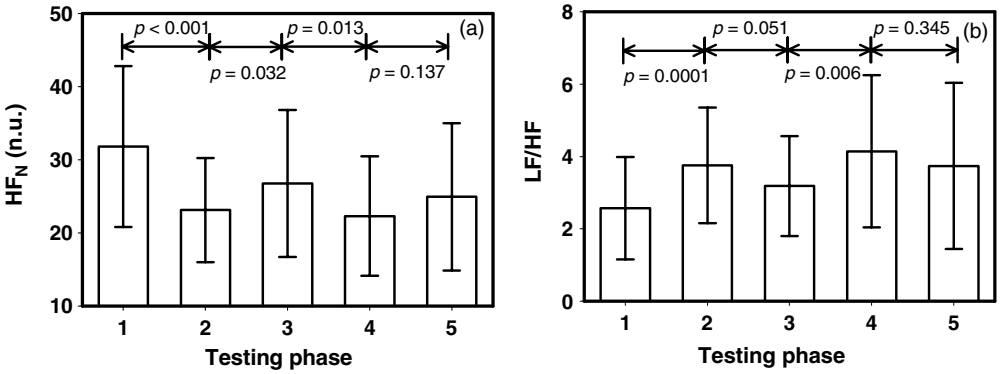

Figure 2. A comparison of (a) normalized HF power and (b) LF/HF ratio during different testing phases. *P*-values shown were obtained from the paired *t*-test. The testing phases are numbered as 1: before PLR test, 2: during LA PLR, 3: during dark adaptation, 4: during DA PLR, 5: after PLR test. The error bars indicate the standard deviation.

Table 5. Correlations between average heart rate measured in different testing phases. The results are represented as Pearson's *r*-value.

	Before test	LA test	DA	DA test	After test
Before test	1.00[a]	0.95[a]	0.91[a]	0.92[a]	0.88[a]
LA test		1.00[a]	0.94[a]	0.95[a]	0.90[a]
DA			1.00[a]	0.93[a]	0.91[a]
DA test				1.00[a]	0.90[a]
After test					1.00[a]

[a] $p < 0.0001$.

(ANCOVA F-value = 6.74 and 8.52, $p < 0.0001$ and $p < 0.0001$). As shown in figure 2, HF normalized power was significantly lower during the two PLR testing phases (both LA and DA tests) than during the three resting periods (before PLR test, dark adaptation and after PLR test), while the LF/HF ratio was significantly higher.

The Pearson product moment correlation did not show any significant correlation between simultaneously measured PLR parameters and HRV parameters ($p > 0.05$, $r < 0.5$ for all the correlations). Further multilinear regression analysis concluded that no significant model could be developed to explain variations in PLR parameters by using HRV parameters, nor vice versa.

Using the exploratory factor analysis with oblique matrix rotation (OBVARIMAX in SAS), the multivariate PLR and HRV dataset was reduced to a system of four factors which were uncorrelated with each other (inter-factor correlations $r < 0.3$). These four factors were determined such that the total variance of the dataset (proportion = 1) can be explained by the resulting system with a reduced dimension and also using the scree plot to identify the last substantial drop which is after factor 4 (Fabrigar *et al* 1999). The factor loading after oblique matrix rotation (OBVARIMAX in SAS) is shown in table 6, where each factor loading represents the correlation between a PLR or HRV parameter and the identified factor. Factors 1 and 2 were mainly attributed to loadings from HRV parameters. The heart rate and time domain HRV parameters (SDNN and rMSSD) had the highest correlation with factor 1, whereas the two frequency domain HRVs, LF/HF and HF_n, had the highest correlation with factor 2. PLR parameters were the major contributors to factors 3 and 4. Factor 3 had a high loading from t_C and t_R, while factor 4 had moderately high correlations with t_L and D_o. The reliability of each variable was evaluated by analyzing 'Cronbach's coefficient of alpha when deleted' with

Table 6. Factors loading for the entire data set by using the factor analysis. The numbers represent correlation between PLR/HRV parameters and the identified factors.

		Factor 1	Factor 2	Factor 3	Factor 4
	RMSSD	−0.93	−0.19	−0.03	0.02
	SDNN	0.95	−0.07	0.00	−0.02
HRV	HR	0.81	0.02	0.06	−0.02
	LF/HF ratio	−0.02	−0.90	0.10	0.01
	HF_n	0.01	0.94	0.03	0.01
	Constriction time	−0.01	0.00	0.93	−0.07
PLR	Redialtion time	0.02	−0.05	0.91	0.09
	Latency	0.02	−0.03	0.04	0.42
	Initial pupil diameter	−0.02	0.02	−0.02	0.34

'PROC CORR'. The results indicated that the heart rate should be removed from factor 1. In addition, a 'Cronbach's coefficient of alpha' of 0.7 was used as the acceptable threshold to evaluate the reliability of the entire factor system. The results indicated that the final solution should only consist of factors 1 and 3, where factor 1 represented the HRV system and factor 3 represented the PLR system. Communalities of selected parameters over the final factor solution were above 0.8 for all the parameters selected in the factor solution. Such consistently high communalities suggested that the sample size of the design had a minimal effect on the factor solution (MacCallum *et al* 1999). The numbers shown in table 6 were calculated by using PLR data obtained at LA 8721.1 cd m^{-2} and HRV data obtained during the LA testing phase. However, the same conclusion was reached for all other testing conditions.

4. Discussion

The PLR parameters obtained in this study are similar to those reported previously (Fan *et al* 2009b) in a similar age group at similar stimulus conditions. Similarly, the time domain HRV parameters obtained in this study are consistent with those reported in previous studies (Umetani *et al* 1998). Our mean HF_n (%) is smaller than that reported by Gamelin *et al* (2008) in boys younger than 11 years in supine position. We think that these differences can be attributed to the seated position used in our study. It is known that HRV measured at supine position has higher HF_n (%) and smaller LF/HF than that measured at standing position (Montano *et al* 1994). The HRV parameters obtained in seated position are in between of those obtained at supine and standing positions (Chan *et al* 2007).

Because 43% of participants were tested in the morning and the rest were tested in the afternoon, we examined the potential effect of different time of day of the test. The results showed no significant difference between the PLR and HRV measurements obtained in children tested in the morning and those tested in the afternoon (t-test, $p > 0.15$). The HRV results (table 4) measured in phases 2 and 3 (figure 1) had longer measurement time (10 min and 15 min, respectively) than the other three phases (5 min each). To investigate whether such a difference may lead to different HRV values, we divided the 10 min LA test period into two 5 min segments and calculated HRV parameters in each segment. The obtained HRV parameters in the two 5 min segments were similar to each other (paired t-test, $p > 0.28$) and were also similar to the one calculated over the entire 10 min period. The same results were obtained for the HRV parameters obtained in the 15 min DA period.

The frequency domain HRV parameters were clearly affected by the PLR testing phases. The mean results (table 4) indicate that HF_n decreased 27.3% and LF/HF increased 45.9%

when going from 'before test' to PLR test. This trend is similar to posture-induced HRV changes that are related to sympathetic activity caused by orthostatic stress (Mukai and Hayano 1995, Montano *et al* 1994, Yeragani *et al* 1993). Because the participant slightly inclined forward (∼15°) during the PLR tests, it is possible that such a posture change led to elevated sympathetic activity due to increased muscle stress. In addition, the PLR test itself may induce some task-related stress. Delaney and Brodie (2000) reported that psychological stress can decrease high-frequency HRV and increase low-frequency HRV. Vagal tone and respiratory sinus arrhythmia (RSA) are the principal contributors to the high-frequency power of HRV, while both vagal and sympathetic tones influence the low-frequency component (Berntson *et al* 1997). Overall the observed changes in frequency domain HRV parameters suggest lower vagal tone and increased sympathetic modulation during the PLR test.

PLR parameters and HRV parameters are categorized as independent and uncorrelated factors according to the factor analysis. The SDNN and RMSSD were closely aligned only with factor 1 and had very low loading on other factors (table 6). Similarly, the constriction time and redilation time were aligned only with factor 3. LF/HF and HF_n were highly aligned with factor 2 with high loading. However, with a smaller loading, PLR latency and initial pupil diameter were best aligned with factor 4. Therefore, the data clearly indicated a lack of association between PLR and HRV. This observation may be attributed to the fact that healthy typically developing children were tested in this study. In this group, many PLR parameters were quite consistent with very small between-subject variation. Notably, the coefficient of variation of PLR latency was $<10\%$, in agreement with the fact that latency did not contribute to the four-factor system derived from the common factor analysis.

The lack of association between PLR and HRV can be further corroborated by the different gender effect in PLR and HRV parameters. In agreement with a previous report by Krishnan *et al* (2009) in a large group of children of similar age range, our data showed that girls had a significantly higher heart rate than boys. A higher heart rate indicates stronger sympathetic influence (Malik *et al* 1996). We observed a trend of smaller resting pupil size in girls although the difference did not reach statistical significance. The same difference was reported by Fan *et al* (2009a) as statistically significant in individuals with a tightly controlled age range. A smaller resting pupil size may suggest a stronger parasympathetic modulation or a weaker sympathetic modulation (Barbur 2004). Summarizing the above comparisons, it is clear that gender effects in PLR and HRV do not completely corroborate with each other.

Although PLR and HRV are part of an integrated ANS, individual neurological pathways behave differently. The natural variations in an individual system may not affect other subsystems. However, this conclusion may not hold true for some disorders associated with ANS dysfunctions where pervasive ANS changes exist in multiple subsystems. For example, significant correlations between PLR and HRV were found in patients with acute schizophrenia (Bär *et al* 2008).

5. Conclusion

We measured PLR and HRV simultaneously in age- and gender-matched healthy children 8–16 years old. The gender effect was observed in both HRV and PLR parameters. However, the significant age effect was observed in HRV parameters but not in PLR. We found that the frequency domain HRV parameters were significantly different in different PLR testing phases, likely due to the psychological or/and physiological stress induced by the PLR tests. Using both the regression analysis and factor analysis, we conclude that variations of PLR and HRV are not associated in healthy children. However, this conclusion might be altered in the case of ANS disorders or other situations when pervasive changes might appear in multiple different

ANS subsystems. Nevertheless, PLR and HRV may provide a complementary assessment of different aspects of the overall autonomic nervous system.

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